Cancer Chemother Pharmacol (2003) 51: 53–57
DOI 10.1007/s00280-002-0547-y

ORIGINAL ARTICLE

**Lili Liu · Takeo Sakaguchi · Tatsuo Kanda
Jiro Hitomi · Yasuhiko Tabata
Katsuyoshi Hatakeyama**

Delivery of interleukin-12 in gelatin hydrogels effectively suppresses development of transplanted colonal carcinoma in mice

Received: 28 May 2002 / Accepted: 25 September 2002 / Published online: 20 November 2002

Abstract *Purpose*: The sustained effects of interleukin-12 (IL-12) incorporated in biodegradable hydrogel on the development of transplanted colon carcinoma were investigated in C57BC/6 N mice. *Methods*: IL-12 was given to the animals incorporated in gelatin hydrogels (GH) which allow its gradual release. As the control administration, IL-12 was given via two subcutaneous injections at an interval of 6 days. Growth of the tumors was evaluated in terms of volume. *Results*: When IL-12 at 500 ng/animal was administered in GH, the tumor volume was decreased 9 and 12 days after administration, and the response was dose dependent in the range 0–500 ng/animal. The effect of IL-12 was stronger in mice treated with GH than in mice treated by subcutaneous injection at the same dose (i.e. 250 ng/animal twice at an interval of 6 days). Feeding behavior and the post-mortem wet weight of several visceral organs were unchanged after IL-12 administration. Serum parameters relative to the liver, heart and kidneys were unaffected by IL-12 treatment. No noticeable toxicity was detected when the dose of IL-12 in GH was increased to 1000 ng/animal. *Conclusions*: The results suggest that IL-12 is effective in suppressing the growth of colon carcinoma, and that delivery of this agent in GH is efficient in the treatment of carcinoma.

Keywords Antineoplastic cytokine · Biodegradable cytokine · Pharmacokinetics · Mouse

L. Liu · T. Kanda · K. Hatakeyama
Division of Digestive and General Surgery,
Department of Regenerative and Transplant Medicine,
Graduate School of Medical and Dental Sciences,
Niigata University, Asahimachi-1,
Niigata 951-8510, Japan

T. Sakaguchi (✉)
Division of Integrative Physiology,
Department of Sensory and Integrative Medicine,
Graduate School of Medical and Dental Sciences,
Niigata University, Asahimachi-1, Niigata 951-8510, Japan
E-mail: sakaguch@med.niigata-u.ac.jp
Tel.: +81-25-2272068
Fax: +81-25-2270755

J. Hitomi
Division of Microscopic Anatomy and Bio-imaging,
Department of Cellular Function,
Graduate School of Medical and Dental Sciences,
Niigata University, Asahimachi-1, Niigata 951-8510, Japan

Y. Tabata
Institute for Frontier Medical Sciences,
Kyoto University, Kyoto 606-8507, Japan

Introduction

Interleukin-12 (IL-12) is a recently discovered heterodimetric cytokine [16, 20], and it has been shown in various animal models of cancers to have tremendous antitumor potential [3, 7]. It has been shown to enhance natural killer and cytotoxic T lymphocyte activity which induce interferon gamma, and to lead to tumor regression [2, 9, 13, 14], but it is usually used for a long time, and adequate sustained levels are required [15, 19, 21]. Biodegradable gelatin hydrogels (GH) have recently been developed, and they have been used for the delivery of medicinal substances [11, 12, 17].

The present study was designed to investigate whether IL-12 gradually released from GH has an effect on the growth of transplanted colon carcinoma in mice.

Materials and methods

Animals

A total of 63 male C57BL/6 N mice (Charles River Japan, Tsukuba, Japan) were used. They were housed and allowed free access to laboratory chow (MF, Oriental Yeast, Osaka, Japan) and tap water throughout the experiments. The room temperature was controlled between 22°C and 24°C with a 12-h/12-h light/dark cycle (lighting from 0800–2000 hours). Estimation of individual body weight and food and water intake, and treatment was done between 1000 and 1200 hours.

Experimental design

Animals bearing no carcinoma were divided into two groups. These animals were treated with IL-12 in GH at 500 ng/animal or with two subcutaneous injections of IL-12 at 250 ng/animal at an interval of 6 days, and these delivery methods were compared by determining the serum concentrations of IL-12. Mice bearing carcinomas were divided into three groups. These animals were treated with GH containing saline (control), with IL-12 incorporated in GH or with IL-12 as subcutaneous injections, respectively, and the differences in relation to delivery method and dose were determined. Mice bearing no carcinoma received a higher dose of IL-12, and the toxic effects were investigated.

Transplantation of tumor

When the mice grew to a weight of about 22 g, tumor transplantation was performed under ether anesthesia by a previously described method [8]. In brief, fragments of tumor, originating in a syngeneic mouse colon carcinoma (CMT-93; Dainippon Pharmaceutical Company, Osaka, Japan) [6], were implanted subcutaneously into the left side of the back. After implantation, the animals were returned to their cages and allowed access to food and water.

Preparation of GH

GH were prepared [11, 12, 17], and it was preliminarily confirmed that IL-12 incorporated into the GH was gradually released into the circulation over several days.

Estimation of tumor size

Tumor volume was estimated using a slide caliper according to the following previously used equation [10, 15, 18]: $0.5 \times \text{length} \times \text{width}^2$. When tumors were growing progressively and the volumes of the implanted tumors were between 80 and 120 mm^3, IL-12 was administered. In vivo activity was evaluated in terms of the percent of the growth of tumors in control animals. Growth of the cancer was determined 3, 6, 9 and 12 days after the first IL-12 administration.

Histological examination

Wet weights of the heart, spleen, liver and kidneys were determined, and paraffin-embedded sections of these tissues stained with hematoxylin and eosin were examined.

Hematological examination

Blood samples were obtained by cardiac puncture 0, 3, 5 and 12 days after cancer transplantation, and the numbers of red blood cells (RBC), white blood cells (WBC) and platelets were estimated using a CELL-DYN 3200 analyzer (Dainabot Company, Tokyo, Japan).

Biochemical analysis

The blood samples obtained by cardiac puncture were cooled immediately in ice water and centrifuged at 2200 rpm for 20 min. Then the separated serum was stored at –20°C until measurement of the following parameters using an autoanalyzer (Hitachi-7070; Hitachi, Tokyo, Japan) [5]: total protein (biuret method), glutamic oxaloacetic transaminase (GOT, ultraviolet method), glutamic pyruvic transaminase (GPT, ultraviolet method), alkaline phosphatase (ALP, Bessy-Lowry method), blood urea nitrogen (BUN, urease ultraviolet method), creatinine (Jaffe method). The concentration of IL-12 was determined using an immunoassay kit (BioSource International, Camarillo, Calif.). The assay kit was specific for IL-12p35 and IL-12p40.

Administration of test agents

IL-12 (Genetics Institute, Madison, N.J.) was given incorported in GH or by subcutaneous injection. GH containing IL-12 were placed subcutaneously into the back adjacent to the implanted tumor. In some animals, GH were placed into the opposite side of the back distant from the implanted tumor. The two subcutaneous injections of IL-12 dissolved in saline were given into the back adjacent to the tumor at an interval of 6 days.

Statistical analysis

The statistical significance of differences among the values was evaluated by ANOVA and Duncan's multiple range test. A significant difference was defined as a difference with a P value < 0.05.

Results

Serum IL-12 concentrations after IL-12 administration in GH and as subcutaneous injections

The serum concentrations of IL-12 were determined 0, 1, 3 and 5 days after administration of IL-12 at 500 ng/animal per 12 days. When IL-12 was given incorporated in GH, an increase in the concentration of IL-12 was seen within 5 days, but when the agent was given by subcutaneous injection, the IL-12 serum concentration did not increase (Fig. 1).

Tumor growth after IL-12 administration

Administration of IL-12 at 500 ng/animal incorporated in GH led to a reduction in tumor volume at 9 and

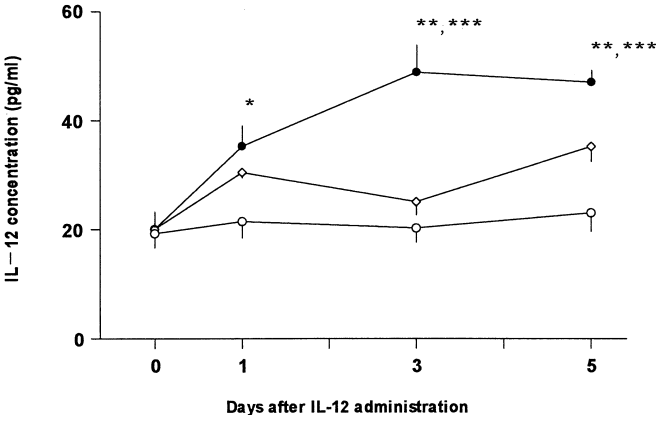

Fig. 1 Serum concentrations of IL-12 after IL-12 administration. IL-12 was given incorporated in GH at 500 ng/animal (*closed circles*) or was given by two subcutaneous injections of 250 ng/animal at an interval of 6 days (*open diamonds*). GH containing no IL-12 was used as the control (*open circles*). Zero indicates the day of the first administration. Values are means ± SEM ($n = 3$). *$P < 0.05$ vs day 0 (*closed circles*); **$P < 0.01$ vs day 0 (*closed circles, open diamonds*). ***$P < 0.01$ vs day 3 (*open diamonds*)

12 days after administration compared to the controls. Although tumor volumes also decreased when the same dose of IL-12 was given by two subcutaneous injections, the decrease was less than following administration of IL-12 in GH (Fig. 2). The response to IL-12 in GH was dose-dependent in the range 0–500 ng/animal. Regression of the tumor was similar when IL-12 at 500 ng/animal was placed into the back adjacent to the tumor or distant from the tumor (Fig. 3).

IL-12 concentration and tumor development

Serum concentrations of IL-12 tended to increase when the dose of IL-12 in GH was increased (Fig. 4), but there was no correlation between the serum IL-12 concentration and tumor volume (data not shown).

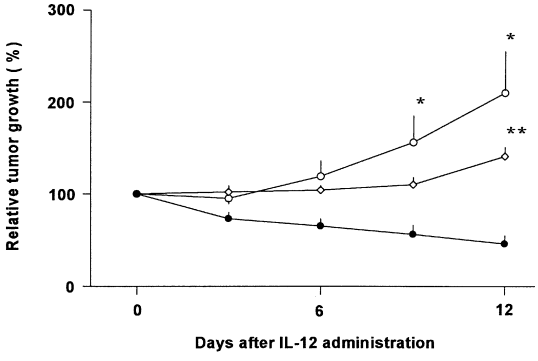

Fig. 2 Relative tumor growth (tumor volume) after IL-12 administration. IL-12 was given incorporated in GH at 500 ng/animal (*closed circles*) or was given by two subcutaneous injections of 250 ng/animal at an interval of 6 days (*open diamonds*). GH containing no IL-12 was used as the control (*open circles*). Zero indicates the day of the first administration. Values are means ± SEM ($n = 6$). *$P < 0.01$ vs IL-12 in GH, **$P < 0.05$ vs control and IL-12 in GH

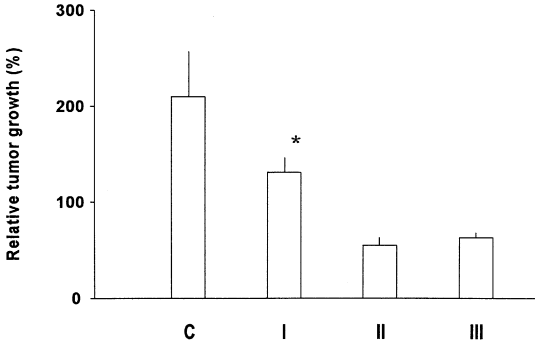

Fig. 3 Relative tumor growth (tumor volume) 12 days after IL-12 administration. Different doses of IL-12 (250 and 500 ng/animal (*I* and *II*, respectively) incorporated in GH were administered into the back adjacent to the tumor, and GH containing no IL-12 was used as the control (*C*). *III* IL-12 at 500 ng/animal incorporated in GH was administered into the opposite side of the back distant from the tumor. Values are means ± SEM ($n = 6$). *$P < 0.05$ vs *C*, *II* and *III*

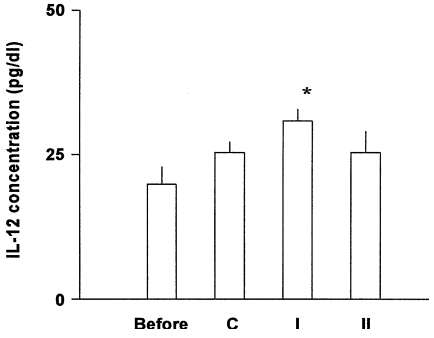

Fig. 4 Serum concentrations of IL-12 before (*Before*) and 12 days after IL-12 administration at (*I*) 250 and (*II*) 500 ng/animal incorporated in GH. GH containing no IL-12 was used as the control (*C*). Values are means ± SEM ($n = 6$). *$P < 0.01$ vs *Before*

Feeding, drinking and body weight gain after IL-12 administration

There were no significant differences among the three groups of mice in food and water intake, or body weight gain during the 12 days after the administration of IL-12 at 500 ng/animal (data not shown).

Wet weight of visceral organs after IL-12 administration

There were no significant differences in the weight of the heart, spleen, liver and kidneys among the three groups of mice 12 days after IL-12 administration at 500 ng/animal (data not shown).

Serum chemistry after IL-12 administration

The concentrations of total protein, GOT, GPT, ALP, BUN and creatinine were unchanged 12 days after administration of IL-12 in GH at 500 ng/animal (Table 1).

General responses of mice to IL-12 at 1000 ng/animal

When IL-12 at 1000 ng/animal was administered in GH, water intake was slightly increased (Table 2). Wet weight of the visceral organs was unaffected, and histological findings were unremarkable (data not shown).

Table 1 Serum chemistry 12 days after administration of IL-12 at 500 ng/animal incorporated in GH. Values are means ± SEM ($n = 6$)

	Control	IL-12 treatment
Total protein (g/dl)	5.5 ± 0.1	5.5 ± 0.1
GOT (IU/l)	406 ± 122	324 ± 135
GPT (IU/l)	60 ± 4	70 ± 5
ALP (IU/l)	350 ± 31	290 ± 25
BUN (mg/dl)	19.5 ± 0.8	18.8 ± 1.4
Creatinine (μg/dl)	12 ± 2	20 ± 7

Table 2 Food intake and hematological parameters 12 days after IL-12 administration at 1000 ng/animal. Values are means ± SEM ($n = 6$)

	Control	IL-12 treatment
Food intake (g/12 days)	39.3 ± 1.1	39.0 ± 0.6
Water intake (ml/12 days)	38.1 ± 1.1	49.2 ± 0.8*
WBC ($\times 10^2/\mu l$)	16.4 ± 1.9	16.0 ± 2.3
RBC ($\times 10^4/\mu l$)	860 ± 21	893 ± 18
Platelets ($\times 10^4/\mu l$)	36.6 ± 14.3	13.8 ± 4.2*

*$P < 0.01$ vs control

Serum chemical parameters were unchanged (data not shown). Hematological examination revealed only a reduction in the number of platelets (Table 2).

Discussion

We found that the administration of IL-12 incorporated in biodegradable GH efficiently suppressed the development of transplanted colon carcinoma. This is consistent with previous findings that IL-12 is active in inhibiting the development of carcinoma [13, 14, 15, 19, 21].

As mentioned above, exogenously administered IL-12 suppresses tumor growth, and an optimal timing of IL-12 administration has been assumed to exist when IL-12 is given by different schedules of administration [19, 21]. In this experiment, continuous release of IL-12 from GH more evidently decreased the growth of the tumor. Therefore, if the time required to complete the processes leading to the antitumor effects were several days, the maintenance of IL-12 at constant levels over time resulting from its administration in GH may result in optimal antitumor immunoactivity.

The inhibitory effect of IL-12 on tumor growth was dose-dependent. This suggests that this cytokine has a direct antitumor effect [2, 9, 13, 14]. The effect of IL-12 on tumor growth may be systemic because the same response was obtained whether it was given adjacent to the tumor or distant from the tumor.

IL-12 is a potent drug, but in many tumors it only produces temporary suppression or slowing of tumor growth, but no cure, and frequently such tumors resume their original rapid growth rate once IL-12 therapy ceases. It has often been demonstrated that the sustained presence of IL-12 either further slows tumor progression or in some cases effects a cure [4]. In this study, the sustained release of IL-12 from GH produced not only delayed tumor growth but also negative tumor growth, so that Il-12 administered by this method might induce a cure.

Serum concentrations of IL-12 were high for several days and depended on the dose of IL-12 incorporated in the GH, but serum IL-12 concentrations were not correlated with the dose 12 days later. It is considered that the surrounding tissues absorb GH and the IL-12 released produces a similar decline regardless of its dose for periods up to 12 days.

Food intake and body weight gain in mice treated with IL-12 were unchanged compared to control mice. This is in accordance with reports that mice can tolerate this therapy well [1, 15]. On the other hand, that water intake increased when the mice received IL-12 at 1000 ng/animal (Table 2) suggests that IL-12 is involved in the mechanism controlling drinking behavior.

The fact that IL-12 had no effect on total protein (Table 1) suggests that the nutritional condition of the animals remained normal. GOT and GPT are transaminase enzymes present primarily in hepatocytes and heart cells. They are released into the blood in larger quantities under conditions of heart or liver damage [1, 15]. In this study there was no significant increase in the concentration of these enzymes. ALP reflecting hepatic biliary dysfunction was also unaffected by IL-12. It appears that there is no toxic effect of IL-12 on the heart or liver. Creatinine is the waste product of muscle metabolism. Its level is a reflection of body muscle mass. Low levels are sometimes seen in people with kidney damage, starvation and/or liver disease [1, 15]. In this study, creatinine and BUN concentrations were unchanged after IL-12 administration. It is likely that the agent has no renal toxicity.

With a reduction in the dose, toxicity would be expected to be reduced. Our observations agree with the reports stating that most toxicities from IL-12 occur at doses far above those used in this study [1]. As shown in Table 2, because the number of platelets was decreased after the administration of IL-12 at 1000 ng/animal, this dose should be not be exceeded. In addition, the threshold concentration above which IL-12 is effective is 250 ng/animal per 6 days.

Subcutaneous tumors, as opposed to tumors of vital organs, are not as well perfused and accessible to immune effector cells such as T cells and natural killer cells, which are critical to the success of IL-12 therapy, but IL-12 gradually released from GH was effective in suppressing the growth of subcutaneous tumors in this study. These findings indicate a strong potential for the use of this method of administration for the treatment of tumors in vital organs [5].

Because clinical tumor therapy was simulated in this study and IL-12 incorporated in GH effectively suppressed the development of carcinoma, we can expect this method of delivery of IL-12 to be advantageous in the treatment of carcinoma without causing severe systemic toxicity.

Acknowledgement The authors are greatly indebted to Mr. J. Higuchi (Division of Surgery) for his skilful technical assistance.

References

1. Brunda MJ, Gately MK (1995) Interleukin-12: potential role in cancer therapy. In: De Vita VT, Hellman S, Rosenberg S (eds) Important advances in oncology, J.B. Lippincott, Philadelphia, p 3

2. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med 178:1223
3. Brunda MJ, Luistro L, Rumennik L, Wright RB, Dvorozniak M, Aglione A, Wigginton JM, Wiltrout RH, Hendrzak JA, Palleroni AV (1996) Antitumor activity of interleukin 12 in preclinical models. Cancer Chemother Pharmacol [Suppl] 38:S16
4. Colombo MP, Vagliani M, Spreafico F, Parenza M, Chiodoni C, Melani C, Stoppacciaro A (1996) Amount of interleukin 12 available at the tumor site is critical for tumor regression. Cancer Res 56:2531
5. Cui X, Sakaguchi T, Shirai Y, Hatakeyama K (1999) Orally administered panax ginseng extract decreases platelet adhesiveness in 66% hepatectomized rats. Am J Chin Med 27:251
6. Franks LM, Hemmings VJ (1978) A cell line from an induced carcinoma of mouse rectum. J Pathol 124:35
7. Fujiwara H, Hamaoka T (1996) Antitumor and antimetastatic effects of interleukin 12. Cancer Chemother Pharmacol [Suppl] 38:S22
8. Funahashi Y, Koyanagi N, Sonoda J, Kitoh K, Yoshimatsu K (1999) Rapid development of hepatic metastasis with high incidence following orthotopic transplantation of murine colon 38 carcinoma as intact tissue in syngeneic C57BL/6 mice. J Surg Oncol 71:83
9. Gately MK, Warrier RR, Honasoge S, Carvajal DM, Faherty DA, Connaughton SE, Anderson TD, Sarmiento U, Hubbard BR, Murphy M (1994) Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-gamma in vivo. Int Immunol 6:157
10. Giuliani FC, Zirvi KA, Kaplan NO (1981) Therapeutic response of human tumor xenografts in athymic mice to doxorubicin. Cancer Res 41:325
11. Hong L, Tabata Y, Miyamoto S, Yamamoto M, Yamada K, Hashimoto N, Ikada Y (2000) Bone regeneration at rabbit skull defects treated with transforming growth factor-β1 incorporated into hydrogels with different levels of biodegradability. J Neurosurg 92:315
12. Kang HW, Tabata Y, Ikada Y (1999) Fabrication of porous gelatin scaffolds for tissue engineering. Biomaterials 20:1339
13. Kikuchi T, Joki T, Abe T, Ohno T (1999) Antitumor activity of killer cells stimulated with both interleukin-2 and interleukin-12 on mouse glioma cells. J Immunother 22:245
14. Kuriakose MA, Chen FA, Egilmez NK, Jong YS, Mathiowitz E, DeLacure MD, Hicks WL Jr, Loree TL, Bankert RB (2000) Interleukin-12 delivered by biodegradable microspheres promotes the antitumor activity of human peripheral blood lymphocytes in a human head and neck tumor xenograft/SCID mouse model. Head Neck 22:57
15. Shah AU, D'Souza MJ (1999) Sustained-release interleukin-12 microspheres in the treatment of cancer. Drug Dev Ind Pharm 25:995
16. Stern AS, Magram J, Presky DH (1996) Interleukin-12 an integral cytokine in the immune response. Life Sci 58:639
17. Tabata Y, Ikada Y (1999) Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials 20:2169
18. Taetle R, Rosen F, Abramson I, Venditti J, Howell S (1987) Use of nude mouse xenografts as preclinical drug screens: in vivo activity of established chemotherapeutic agents against melanoma and ovarian carcinoma xenografts. Cancer Treat Rep 71:297
19. Tahara H, Zeh HJ 3rd, Storkus WJ, Pappo I, Watkins SC, Gubler U, Wolf SF, Robbins PD, Lotze MT (1994) Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. Cancer Res 54:182
20. Trinchieri G (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen- specific adaptive immunity. Annu Rev Immunol 13:251
21. Zitvogel L, Tahara H, Robbins PD, Storkus WJ, Clarke MR, Nalesnik MA, Lotze MT (1995) Cancer immunotherapy of established tumors with IL-12. Effective delivery by genetically engineered fibroblasts. J Immunol 155:1393